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Autophagosome Maturation and Fusion

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Abstract

Macroautophagy, or simply autophagy, is a degradative pathway that delivers cytoplasmic components, including cytosol and organelles, to the lysosome in double-membrane vesicles called autophagosomes. This process is initiated at the pre-autophagosomal structure or phagophore assembly site and involves a number of highly conserved autophagy-related proteins. These support the generation and conversion of an open membranous cistern known as the phagophore or isolation membrane into a closed autophagosome. Within this review, we will focus on recent insights into the molecular events following the sealing/completion of an autophagosome, which lead to its maturation and subsequent fusion with endosomes/lysosomes.

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Introduction

Autophagy is a general catabolic pathway, by which cells mobilize nutrients such as amino acids through the degradation of long-lived proteins, dysfunctional complexes and organelles, and invading pathogens [49,54,70]. In addition, it is used to adjust the organelle repertoire of cells in order to adapt to metabolic requirements or target specific unwanted proteins to the lysosome. Autophagy is divided into selective types of autophagy, which eliminate distinct cargos and make use of the so-called autophagy receptors, and into nonselective, bulk autophagy, which sequesters portions of the cytoplasm in an apparent non-specific manner [50,99].

A conserved group of 16 autophagy-related (ATG) proteins has been identified as the core machinery for autophagosome biogenesis. Initially discovered and characterized in yeast [33,108,111], homologs are now known in all eukaryotes [49,54,70]. These Atg proteins fall into five general protein complexes: (1) the Atg1/ULK kinase complex, (2) the Atg12 conjugation system, (3) the Atg8/LC3 conjugation/deconjugation system, (4) the phosphatidylinositol 3-kinase complex (PI 3-kinase complex), and (5) the

Atg9/ATG9L1 cycling system [49,54,70]. One potent inducer of autophagy is nutrient starvation. Under normal and nutrient-rich growth conditions, the lysosomal target of rapamycin complex 1 inhibits autophagy by phosphorylation of subunits of the Atg1 complex [41,80]. Conversely, amino acid deprivation inhibits target of rapamycin complex 1, and subsequent dephosphorylation of the Atg1 complex strongly activates autophagy. Autophagy thus enables cells to mobilize amino acids that are needed for survival under these conditions. However, other nutritional, stress and developmental signals can also trigger autophagy [7,26,123].

Autophagy is initiated at one or several pre-autophagosomal structure or phagophore assembly site (PAS), where the Atg machinery assembles upon autophagy induction [44,102,103]. The PAS localizes proximal to the ER [29,101], and in mammals, these sites are often referred to as omegasomes [2,54]. Here, membranes of probably different sources contribute to the formation of a phagophore or isolation membrane, a disk-like structure that expands, rounds up into a cup-shaped structure, and eventually closes around its cargo to become an autophagosome [49,54,70]. Several studies imply that the ER exit

sites are essential for autophagy and proximal to the PAS and that COPII-coated vesicles contribute to autophagosome formation [27,29,96,101]. Other studies have also implicated recycling endosomes, the Golgi apparatus, plasma membrane, mitochondria, and endoplasmic reticulum (ER)-mitochondrial contact sites as critical membrane sources and/or landmarks for the assembly of the ATG machinery [5,32,47,59,83,89,90,113]. Atg9/ATG9L1 is the only transmembrane protein among the core Atg proteins [55,79,124]. Atg9-containing vesicles cycle between the PAS and the Golgi/endosomes, and they appear to contribute at least in part to the membranes for the nucleation of the phagophore [61,84,92,122]. Phagophore elongation results eventually in a fission event at the extremities of this growing cistern, which generates a closed autophagosome with an inner and outer membrane. The proteins involved in this process are not yet clear. Atg9 and its interacting partners Atg2 and Atg18 are found at the edges of the expanding yeast phagophore, and the members of the small ubiquitin-like Atg8/LC3 protein family (see also below) have been implicated as possible closure factors [29,101,110,119]. As the sealing process remains incompletely understood, we will primarily discuss in this review the steps following this event, that is, maturation and fusion, although we will elude to possible earlier events whenever possible.

Autophagosomes that have been just sealed still have almost all the Atg proteins on their surface, which need to be recycled for reuse. One or more of these proteins may also act as inhibitors on the recruitment and/or activation of the fusion machinery. Maturation of autophagosomes, defined as the removal of all Atg proteins from their surface that initiated after the closure of the phagophore, requires, for example, both PI3P turnover and members of the Atg8/

LC3-specific Atg4 protease family (discussed below). This transition also involves the acquisition of the fusion machinery, which needs to be active at the right time, that is, only after the closure of the phagophore, to avoid premature fusion of incomplete structures. Below, we summarize our current knowledge on both processes—maturation and fusion of autophagosomes—by comparing yeast with metazoan cells.

Maturation of Autophagosomes

Several model systems have been used to dissect the molecular mechanisms that underlie autophagy. The yeast *Saccharomyces cerevisiae* has been a prime resource of knowledge due to its relative experimental simplicity and its initial exploitation to identify the Atg machinery [76]. Therefore, we will first discuss yeast in the subsequent chapters, followed by the insights obtained for mammalian cells.

Early during the first steps of autophagy, two modifications are the key in defining an autophagosome as a unique organelle: the conjugation of the ubiquitin-like protein Atg8/LC3 to phosphatidylethanolamine (PE) and the generation of phosphatidylinositol-3-phosphate (PI3P) by the autophagy-specific PI 3-kinase complex [49,54,70]. Several Atg proteins take advantage of the PI3P pool on autophagosomal membranes for their efficient binding, and other autophagosome-associated proteins have LC3/Atg8 interacting regions motifs [50,99]. Removal of PI3P and Atg8/LC3 after the closure of the phagophore thus very likely destabilizes other Atg proteins and thus enables their efficient dissociation and recycling (Fig. 1).

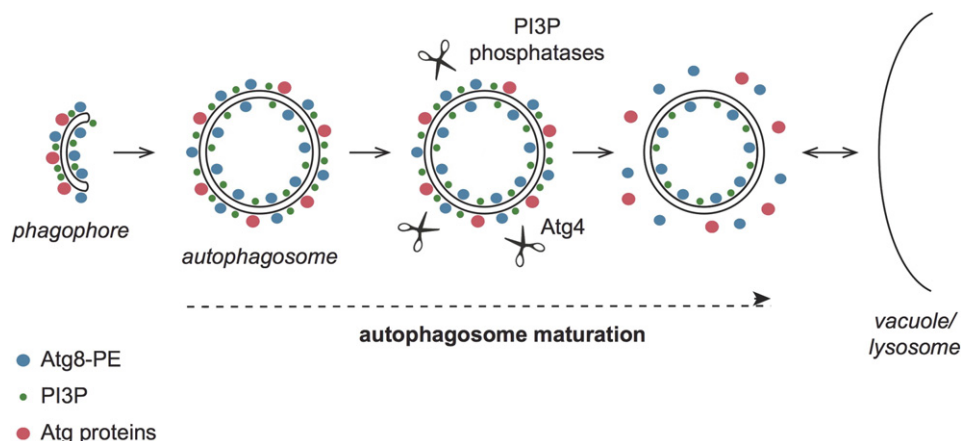


Fig. 1. Simplified overview of autophagosome maturation. Phagophores are positive for numerous Atg proteins, PE-conjugated Atg8 (in metazoans LC3-II) and PI3P, and upon closure, they form double-membrane autophagosomes. Autophagosome maturation is characterized by the dissociation of most Atg proteins, which also involves the removal of Atg8 and PI3P from the surface of autophagosomes by Atg4 (in metazoans ATG4A to ATG4D) and Ymr1 (in metazoans MTMRs), respectively, prior to their fusion with degradative compartments. For details, see text.

Maturation of yeast autophagosomes

Atg8 function requires its constitutive post-translation processing at the C terminus by the protease Atg4 [46]. The exposed C-terminal glycine is subsequently conjugated to PE on phagophore membranes by a complex ubiquitin-like E3 ligase system [36,69]. Interestingly, Atg4 is also involved in Atg8 recycling from membranes through the hydrolysis of the bond between Atg8 and PE [46]. To understand the relevance of the second cleavage in autophagy, a truncated form of Atg8 that exposes the C-terminal glycine, named Atg8 Δ R, has been expressed in *atg4* Δ knockout strains. Even though the cells generate autophagosomes, as they are able to conjugate Atg8 to PE, they still require Atg4 for efficient autophagosome biogenesis, and subsequent fusion with the vacuole is strongly impaired [73,75,126]. This suggests either that the presence of the remaining Atg proteins in complex with Atg8 inhibits fusion or that the amount of readily available Atg8 is limiting for efficient de novo autophagosome formation. If Atg8 removal is important for autophagosome fusion with the vacuole, then Atg4 function must be tightly regulated. A simple model would predict the existence of two pools of Atg4. Cytosolic Atg4 would constitutively process Atg8 right after translation, whereas PAS-localized Atg4 may process Atg8-PE. Interestingly, unlike all other Atg proteins, Atg4 is not found at the PAS [75], and therefore, it must be recruited to this location. An alternative scenario that is not mutually exclusive is that the Atg8-PE pool on the growing phagophore is somehow protected from the action of cytosolic Atg4, and at the autophagosome completion, this inhibition is relieved. It is also possible that Atg4 activity is counterbalanced by intense Atg8-PE generation during the phagophore elongation and may prevail once Atg8 conjugation is declining the completion of the autophagosome.

A second important hydrolase in autophagosome maturation is the PI3P-specific MTMR-like Ymr1 protein, which has redundant function with the general phosphoinositide phosphatases Sjl2 and Sjl3 in the endosomal system [85]. Deletion of Ymr1 leads to a severe impairment of autophagy, which is exacerbated if combined with Sjl2 and/or Sjl3 depletion [10]. Under these conditions, closed autophagosomes accumulated in the cytoplasm, suggesting that the efficient removal of PI3P is needed to make them fusion competent [10,14]. The simplest mechanistic model is that PI3P turnover leads to the release of those Atg proteins that need to bind this lipid to associate to autophagosomal membranes. In agreement, Ymr1 and Sjl3 could be found at the PAS upon induction of autophagy [10]. These results were confirmed by detailed and impressive freeze-fracture analyses of yeast autophagosomes, which visualized the lipid directly [14]. As Ymr1 can be detected at the PAS [10],

it is not yet known how its activity is controlled during the formation and maturation of the autophagosome.

Maturation of mammalian autophagosomes

The role of the members of the Atg4 protease family in autophagosome maturation in mammalian cells remains to be elucidated, but few reports indicate that they might play a similar function as their yeast counterpart. For example, ATG4B and ATG4D activities are important for autophagosome fusion with degradative compartments in human erythroblasts during differentiation [4].

Although several PI3P phosphatases of the MTMR family such as Jumpy/MTMR14, MTMR3, MTMR6, and MTMR7, have been involved in the initial steps of autophagy, some more clearly than others [21,71,105,114], it has only been recently revealed that a phosphatase belonging to the same family, that is, MTM-3, is required for autophagosome maturation and fusion in *Caenorhabditis elegans* [120]. Identical to yeast Ymr1, MTM-3 acts downstream of the autophagy machinery and prior to the one involved in fusion [120]. In this regard, it is important to note that mammalian autophagosomes are also decorated with PI3P similar to those of yeast, even though the yeast autophagosomes have a far higher luminal PI3P content than those in mammals [14,88]. Future studies will need to address whether this is due to the relative difference in PI3P phosphatase activity between yeast and metazoan cells or whether it can be attributed to other factors involved in PI3P generation and turnover. In this regard, it must be noted that the conversion of PI3P into phosphatidylinositol-3,5-bisphosphate by yeast Fab1 and mammalian PIKfyve kinases is an important mechanism to dissipate PI3P during endosome maturation [65,82]. Interestingly, Fab1/PIKfyve is required for metazoan autophagy. Ablation of Fab1 in *Drosophila* and *C. elegans* causes an accumulation of autophagosomes and amphisomes [78,95]. Similarly, treatment of mammalian cells with PIKfyve inhibitors also leads to an accumulation of autophagosomes [18,40,62]. However, it remains unclear whether the observed defects are due either to an impairment of autophagosome maturation or to the fusion of these vesicles with endolysosomal compartments. Nonetheless, they highlight the possible existence of a more complicated scenario for PI3P turnover in metazoan cells.

Summary

Autophagosomal closure and maturation depend on an order of yet minimally understood steps. Maturation, in particular, appears to require the removal of PI3P and Atg8/LC3 by phosphoinositide phosphatases and possibly other factors and by members of the Atg4 protease family, respectively (Fig. 1). This probably leads to the release of a large

part of the autophagosome-associated Atg proteins. Loss of Atg proteins is likely paralleled by the activation of the fusion machinery, which we will discuss next.

Fusion of Autophagosomes with Lysosomes

Autophagosomes form *de novo*, and thus, they need to acquire the machinery to fuse with lysosomes at the beginning and/or during the course of their biogenesis. The overall recruitment strategies and the implicated fusion machinery seem to differ between metazoan and yeast cells, and therefore, they will be discussed sequentially.

Fusion of yeast autophagosomes with vacuoles

Autophagosome fusion with vacuoles requires the RAB7-like Ypt7 protein, its interacting homotypic vacuole fusion and protein sorting (HOPS) tethering complex, and SNAREs, and thus, it does not seem to differ much from what was known about endosome–vacuole fusion (Fig. 2, left panel) [3,53]. Ypt7, as all Rab GTPase, requires a guanine nucleotide exchange factor (GEF) for activation, and in its specific case is the

Mon1–Ccz1 complex, which localizes to endosomes and vacuoles [52,81,115,116]. Ypt7-GTP binds to the HOPS complex, a hexameric tethering complex with two Rab binding sites at opposite ends of its elongated structure [6,98,121]. The HOPS complex also recognizes selected SNAREs and could therefore also bridge membranes by binding to Ypt7 and SNAREs [51,58,60,100]. Recent data on autophagy in *Drosophila* revealed that Mon1–Ccz1 also localizes with RAB7 onto autophagosomes [34]. It is thus conceivable that also yeast Mon1–Ccz1 loads Ypt7 onto autophagosomes (Fig. 2, left panel).

Several early studies identified the SNARE machinery for the fusion of late endosomes and autophagosomes with vacuoles. Three Q-SNAREs, Vam3, Vti1 and Vam7, and the R-SNARE Ykt6 are essential for both processes [17,20,25,37]. While Vam3 and Vam7 localize primarily to vacuoles, Vti1 and Ykt6 also function in the endocytic pathway and at the Golgi. Recent data suggest that during autophagosome–vacuole fusion, the SNARE Vam7 interacts with the Atg17–Atg31–Atg29 trimer, which is part of the Atg1 kinase complex and acts at the early steps of autophagosome biogenesis [57]. A mutant Atg17 with a Vam7 binding defect shows a reduction in the fusion of autophagosomes with vacuoles, suggesting a direct crosstalk of Atg17 with the fusion machinery.

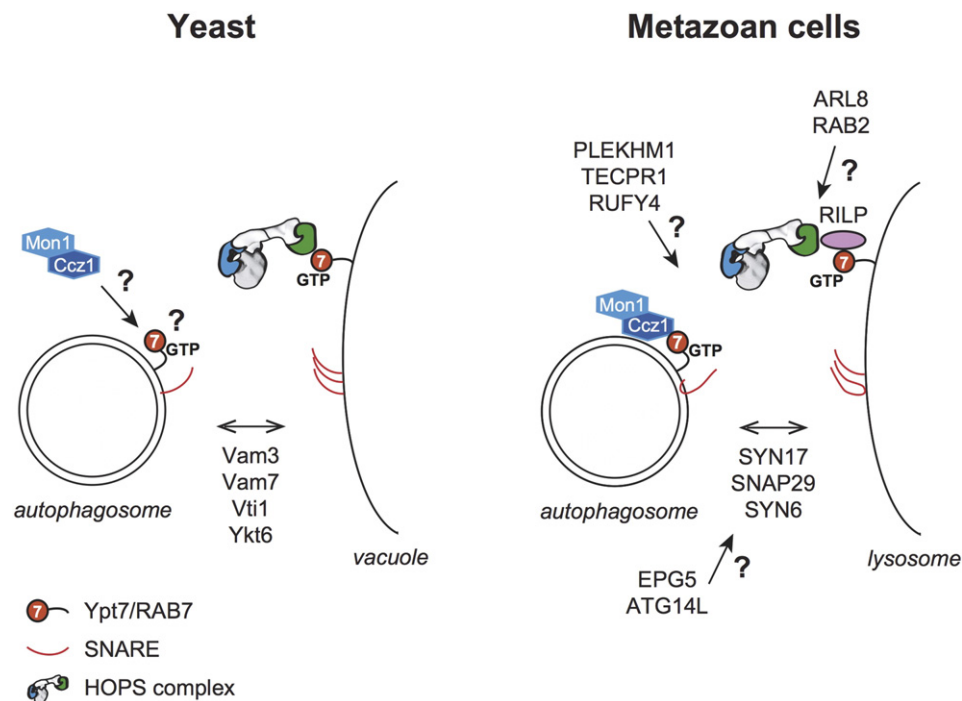


Fig. 2. Fusion of autophagosomes with vacuoles and lysosomes. Comparison of yeast (left) and metazoan (right) autophagosome fusion with vacuoles and lysosomes, respectively. The position of the HOPS tethering complex on vacuoles/lysosomes and its interaction with possible interactors are indicated. Involved SNAREs (red lines in the figure) are listed below, although their precise distribution is only partially known. Multiple factors have direct or indirect functions in fusion and are further discussed in the text.

Vam7 is unusual SNARE as it lacks a transmembrane domain and binds via its N-terminal PX domain to PI3P [12]. This interaction of Vam7 with Atg17 could occur on the surface of either vacuoles or autophagosomes. Given that Atg1 and Atg13 interact with Atg17 and have been found on the vacuole during the selective type of autophagy [109], we find it more plausible that the Vam7–Atg17 crosstalk occurs on the vacuole limiting membrane, but this remains to be proven experimentally. SNAREs need to be membrane-anchored via a transmembrane domain, if they function as the sole SNARE on a vesicle surface. As Vam7 lacks this domain, it will need the assistance of other SNAREs on the autophagosomal surface for efficient fusion with vacuoles. Which SNARE is needed on the yeast autophagosome for fusion, and when and how it is recruited, remains currently unresolved.

Factors required for the fusion of metazoan autophagosomes with degradative compartments

Whereas the overall Rab and SNARE requirements in yeast for the fusion of autophagosomes seem to be known, multiple auxiliary factors have been identified in metazoan cells (Fig. 2, right panel). Importantly, metazoan autophagosomes fuse with late endosomes to form amphisomes [23,24,91,95] before delivering content lysosomes via a kiss-and-run process, which results in the formation of autolysosomes [39]. Efficient fusion between lysosomes and autophagosomes requires the coordinated transport of these two organelles to the perinuclear area [48,87]. Starvation causes an increase in the intracellular pH, which induces lysosome relocation to the perinuclear area [35,48]. Under the same conditions, newly formed autophagosomes are transported to the same cell region of the cell by an interaction with microtubules [72]. Subsequent recycling processes, such as the retrieval of resident hydrolases by tubulation and fission of the lysosomal surface, have been identified, and they are needed to regenerate lysosomes from autolysosomes [22,93,94,125]. It thus seems that the spatial positioning of autophagosomes and lysosomes is an important additional cue for fusion apart from the acquisition of the fusion machinery per se.

As in yeast, metazoan autophagosomes require RAB7 for their fusion with late endosomes/lysosomes [9,31,34]. At least in *Drosophila*, RAB7 and its GEF MON1–CCZ1 are also found on autophagosomes [34]. MON1–CCZ1 localization to autophagosomes requires PI3P, although it does not depend on the early endosomal RAB5 protein unlike its localization to endosomes [15,34,45]. All subsequent steps seem to differ somehow between organisms. In contrast to yeast, the metazoan HOPS complex does not seem to bind RAB7 directly, but instead, it interacts with the small GTPases ARL8 and RAB2 and with the

RAB7-interacting lysosomal protein [28,43,56,112]. Both ARL8 and RAB7-interacting lysosomal protein have been found on lysosomes, whereas RAB2 has been localized to the Golgi and secretory granules [11,30]. Furthermore, PLEKHM1 has been identified as a direct multivalent interactor of both LC3 and the HOPS complex and also regulates the fusion between autophagosomes and lysosomes [66,77]. Through PLEKHM1, *Salmonella* modulates the RAB7-dependent recruitment of host membranes, which is required to establish its replicative vacuoles [67]. Another protein with a potential role in autophagosome–lysosome fusion is TECPR1, which interacts with subunits of the Atg8 E3 ligase complex, that is, the Atg12–Atg5 conjugate, and PI3P [13]. Absence of TECPR1 results in the accumulation of autophagosomes, which apparently cannot fuse efficiently with lysosomes. Recently, the *C. elegans* protein EPG5 was identified as another RAB7 interactor on endosomes/lysosomes, which seems to tether autophagosomes and lysosomes and promote SNARE assembly [117]. EPG5 is also present in the human genome, and its mutation causes the VICI syndrome, which is associated with a degradative defect in autophagy [16]. Finally, RUFY4 was identified as a positive regulator of autophagy by promoting both autophagic flux and the tethering of autophagosomes with lysosomes [107]. RUFY4 contains both a RUN domain for interaction with small GTPases, which is also present in PLEKHM1 [104], and a PI3P-interacting FYVE domain. The protein appears to be specific for immune cells but may have a more broad function also in other higher eukaryotes. It has been speculated that RUFY4 could compete with RUBICON as a negative regulator of endosome–lysosome fusion [64], as both proteins might compete for RAB7.

Although the crosstalk between all these factors still remain to be dissected at the molecular level, their study has revealed that not all the Atg proteins and PI3P are completely cleared from the surface of sealed autophagosomes. One speculative idea is that there could be microdomains on the surface of autophagosomes where, possibly protected by binding partners, those factors are not released. Alternatively, it cannot be excluded that maturation and fusion events take place simultaneously. Other scenarios, however, are also possible, including a novel recruitment of these factors onto autophagosomal membranes. In this context, it is interesting to note that a recent publication has shown that the members of the Atg8/LC3 protein family coordinate and mediate autophagosome–lysosome fusion [77]. However, there are evidences that this is due to the failure of recruiting fusion modulators like PLEKHM1 [66,77] and an impairment of autophagosome sealing [110,119]. It thus remains unclear whether the fusogenic properties of the Atg8/LC3 proteins [74,118] play a role in the direct fusion between autophagosomes and lysosomes.

Fusion of metazoan autophagosomes depends on the SNAREs Syntaxin 17 (SYN17), SNAP29, and VAMP7 or VAMP8 [38,106]. Among these, SYN17 was localized to autophagosomes and interacts with the HOPS complex to mediate fusion with lysosomes [42,106]. SYN17 is unusual in that it is bound to membranes via a hairpin transmembrane domain, which may interact via an intramolecular glycine-zipper motif along the two faces of the hairpin [38]. This may enable SYN17 to associate late with autophagosomes, even though the recruitment mechanism remains unclear. Interestingly, SYN17 associates with autophagosomes and has also been found on mitochondria, where it has been implicated in both the fusion of mitochondria-derived vesicles with lysosomes and mitochondrial fission [1,68]. It is thus possible that SYN17 marks multiple membranes for their fusion with lysosomes. Recent studies suggest that even early-acting factors like ATG14L, a subunit of the PI 3-kinase complex, may promote efficient SNARE assembly during fusion between autophagosomes and lysosomes [19]. This function of an early-acting Atg protein would be reminiscent of Atg17, which also interacts with the SNARE Vam7 during yeast autophagosome fusion with vacuoles [57].

Summary

Autophagosomes appear to acquire RAB7 with the help of the GEF MON1–CCZ1 on their surface. As in yeast, the HOPS complex seems to act downstream of RAB7, although its multiple interactors make it presently challenging to assign a specific function, as other proteins like EPG5 or PLEKHM1 also have important contributing functions (Fig. 2). It is noteworthy that the HOPS complex binds the autophagosomal SNARE SYN17 [42,106], suggesting that the overall SNARE chaperoning function of this complex may be conserved.

Outlook

In both yeast and metazoan cells, autophagosome maturation appears to follow a conserved process that leads to the release of autophagosome-specific marker proteins such as Atg8/LC3 and PI3P. Although the involved hydrolases have been identified, neither their recruitment nor their regulation mechanisms are known. This is particularly challenging for Atg4, which acts both as an activator of Atg8/LC3 and as a recycling factor. We speculate that the microenvironment on the autophagosomal membrane, which is determined by the associated Atg proteins and lipids, controls both Atg4 and MTMR phosphatase functions during autophagosome maturation. Whether PI3P phosphatases and Atg4 functions are interdependent is so far not clear. Importantly, PI3P and lipidated LC3 are also present on other subcellular compartments.

For example, these two factors are simultaneously on phagosomes during LC3-associated phagocytosis [63,97] or on endosomes in specific secretory cells such as goblet and Paneth intestinal cells [8,86]. Are PI3P phosphatases and Atg4 proteins engaged in these pathways via similar mechanisms or is their local triggering regulated differently? Future studies are needed to provide an answer to this question.

A sort of similar regulation may apply for MON1–CCZ1, RAB7, and/or associated SNAREs like SYN17, which appear to be present on autophagosomes early during the biogenesis of these vesicles, yet they function late. A future challenge will be the dissection of such regulatory events at the molecular level, which will probably require new assays and novel information on the interactions between the maturation and fusion machineries on autophagosomes. The understanding of the fusion of autophagosomes with lysosomes in metazoan cells, where several different proteins modulate fusion, remains particularly challenging. Here, it will be critical to distinguish those proteins that execute tethering and fusion from those that assist or function only in specific tissues or allow the potential interaction with other cellular components such as the cytoskeleton. Furthermore, it seems that the spatial positioning of lysosomes and autophagosomes to the perinuclear region is coupled to the efficiency of fusion, although it remains to be clarified how fusion efficiency is linked to the positioning of lysosomes in the cell.

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membrane fusion

Abbreviations used:

Atg, autophagy-related; PI 3-kinase complex, phosphatidylinositol 3-kinase complex; PAS, phagophore assembly site; PE, phosphatidylethanolamine; PI3P,

phosphatidylinositol-3-phosphate; MTMR, myotubularin; GEF, guanine nucleotide exchange factor; ER, endoplasmic reticulum; HOPS, homotypic vacuole fusion and protein sorting; SNARE, soluble N-ethylmaleimide sensitive fusion protein receptor.

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